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Swarming Differentiation and Swimming Motility in *Bacillus subtilis* Are Controlled by *swrA*, a Newly Identified Dicistronic Operon

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The number and disposition of flagella harbored by eubacteria are regulated by a specific trait successfully maintained over generations. The genes governing the number of flagella in *Bacillus subtilis* have never been identified, although the *ifm* locus has long been recognized to influence the motility phenotype of this microorganism. The characterization of a spontaneous *ifm* mutant of *B. subtilis*, displaying diverse degrees of cell flagellation in both liquid and solid media, raised the question of how the *ifm* locus governs the number and assembly of functional flagella. The major finding of this investigation is the characterization of a newly identified dicistronic operon, named *swrA*, that controls both swimming motility and swarming differentiation in *B. subtilis*. Functional analysis of the *swrA* operon allowed *swrAA* (previously named *swrA* [D. B. Kearns, F. Chu, R. Rudner, and R. Losick, Mol. Microbiol. 52:357–369, 2004]) to be the first gene identified in *B. subtilis* that controls the number of flagella in liquid environments and the assembly of flagella in response to cell contact with solid surfaces. Evidence is given that the second gene of the operon, *swrAB*, is essential for enabling the surface-adhering cells to undergo swarming differentiation. Preliminary data point to a molecular interaction between the two gene products.

Flagellated bacteria, when grown over solid surfaces, can adapt their locomotion machinery to achieve a specialized form of flagellum-driven motility called swarming. While swimming in liquid environments is brought about by individual cells independently perceiving chemical signals that trigger adaptive chemotactic responses, swarming is characterized by a multicellular movement of bacteria that migrate above solid substrates in groups of tightly bound cells (15, 16). This type of locomotion is strictly dependent on the ability of surface-adhering bacteria to undergo a differentiation process characterized by the production of specialized swarm cells, longer and more flagellated than planktonic cells. Swarm cells are able to revert into the short oligoflagellated swimmer cells when the advancing front of swarmers stops migrating (1, 7, 9, 12, 14, 15, 25, 36). Swarming, therefore, is regarded as a behavioral response to the surface, which provides flagellated bacteria with the ability to act as a multicellular population rapidly colonizing nutrient-rich solid substrates (38). Swarming of many different gram-positive and gram-negative bacteria has been described, and it is increasingly recognized in several soil *Bacillus* species (9, 20, 21, 36, 37).

Several environmental signals have been described that affect the transition from swim to swarm cells in diverse bacterial species (15). Collective differentiation of swimmers into swarmers has been reported to be critically dependent on cell density signals (6, 24), and a role for surfactants in swarming differentiation has been proposed, as they may be secreted in response to cell population density signals (24). However, although the forward movement of swarm cells is often encased within a wetting slime of diverse composition (22, 39), surfactants are more likely to facilitate migration of swarm cells (13) rather than induce swarming differentiation (4, 21, 37). Signals evoking a chemotactic response have also been suggested to play a role in swarming differentiation; nevertheless, chemotaxis itself is not required for the outward migration of swarm cells, and it is still not known whether the chemotaxis sensory system plays a role in swarming differentiation (2, 36). The essential requirement for initiating swarming differentiation in all bacteria studied up to the present is bacterial contact with a solid surface (19, 26). Little is known about the mechanism whereby bacteria perceive the surface; in *Vibrio parahaemolyticus*, inhibition of flagellar rotation is thought to act as a mechanosensory signal of surface sensing (19, 28).

Genes expressed exclusively in swarm cells have not been identified, but global gene expression profiles show that expression of at least one-third of the functional genome is differentially regulated during transition from swimmers into swarmers in *Salmonella enterica* serovar Typhimurium (40). Nothing is known about the molecular mechanisms involved in signal transduction pathways channeling the surface sensing into specialized gene expression that leads to swarm cell differentiation.

The behavior of a *Bacillus subtilis ifmP* mutant (PB5249) isolated from the laboratory strain PB1831 was recently described (37). Like other laboratory strains tested for swarming motility, PB1831, although motile, was nonswarming and did not produce flagella when transferred from liquid onto solid

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TABLE 1. *B. subtilis* strains used in this study

Strain	Parent	Genotype	Source or reference
PB1831[a]		*trpC2 pheA1 swrAA*	J. A. Hoch
PB5249		*trpC2 pheA1 swrAA*$^+$	37
PB5340	PB1831	*trpC2 pheA1 swrAA*$^+$ Kan	This study
PB5334	PB5249	Δ*swrAA*::Kan	This study
PB5336	PB5249	Δ*swrAA* Δ*swrAB*::Kan	This study
PB5349	PB5336	Δ*swrAA* Δ*swrAB*::Kan *amyE*::*swrAA*$^+$ *cat*	This study
PB5369	PB5336	Δ*swrAA* Δ*swrAB*::Kan *amyE*::*swrAA*$^+$ *swrAB*$^+$ *cat*	This study

[a] Laboratory strain harboring a 9-bp A · T *swrAA* allele; strain was previously designated JH642.

media. In contrast, PB5249 exhibited an increased number of flagella in liquid media and the ability to switch from swimming to swarming motility when propagated on culture media of increasing viscosities. The *ifmP* mutation was found to correspond to that recently identified by Kearns et al. (21) in *yvzD*; the mutated gene was named *swrA* since it led to impairment in swarming differentiation. In the present investigation, we describe a new dicistronic operon to which the *swrA* gene belongs. For this reason, the *swrA* gene is renamed *swrAA* and the second gene in the operon, *yvjD*, is designated *swrAB*. We demonstrate that the two genes are both required for swarming differentiation; however, while *swrAA* is absolutely necessary for flagellation on solid surfaces, its activity alone does not allow the flagellated cells to undergo swarming differentiation. The fully swarming phenotype is complete only when *swrAB* is present. Evidence is given to suggest that an interaction occurs between SwrAA, a soluble intracellular protein, and the plasma membrane-bound SwrAB.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *B. subtilis* strains used in this study and their origins are listed in Table 1. All strains were grown at 37°C either in tryptone-NaCl (TrB; tryptone, 1%; NaCl, 0.5%) or in Luria-Bertani (LB) broth. Media were routinely solidified with 1.5% agar unless otherwise specified. The *Escherichia coli* strains DH5α (*supE44 lacU169* [Δ80 *lacZΔM15*] *hsdR17*[r_K^- m_K^+] *recA1 endA1 gyrA96 thi-1 relA1*) and BL21 (F^- *ompT hsd*S_B[r_B^- m_B^-] *gal* [*dcm*]) were grown in LB broth. When necessary, media were supplemented with 100 μg ml^{-1} ampicillin, 2 μg ml^{-1} kanamycin, or 5 μg ml^{-1} chloramphenicol. When appropriate, 1 mM IPTG (isopropyl-β-D-thiogalactoside) was added to the media.

Strain construction. A number of plasmid derivatives were constructed in several steps for the functional analysis of the *swrA* operon. A starting point for subsequent constructions was plasmid pCC1, obtained in the following way. A DNA segment corresponding to the 3′ region of *ctpB* and part of the intergenic region between *ctpB* and s*wrAA* was amplified by using primers vjB-F (KpnI site) and vjB-R (XhoI site) (Table 2). This fragment was inserted between the KpnI/XhoI sites of pJM114 (31), downstream of the kanamycin resistance gene, thus generating pCC1. To transfer the 8-bp A · T sequence of gene *swrAA* into a PB1831 genetic background, a region including the entire *swrAA* gene was amplified from chromosomal DNA extracted from PB5249 by using primers vzD-F (EcoRI site) and vzD-R (BamHI site) (Table 2). The amplified product was cloned upstream of the kanamycin resistance gene of pCC1, thus producing pCC8A. Following linearization, pCC8A was used to transform strain PB1831 and transformants were selected for kanamycin resistance. The selected clone, PB5340 (Table 1), was confirmed by chromosomal DNA sequencing.

Plasmid pCCΔ*ifmA* was generated from pCC1 in two steps. The region upstream of *swrAA* was amplified by using primers vzD-F (EcoRI site) and A-rev (XhoI site) (Table 2), and the *swrAB* coding region was amplified by using primers B-for (XhoI site) and C-rev (SmaI site) (Table 2). The PCR products were digested with EcoRI/XhoI and XhoI/SmaI, respectively, and then ligated. The ligation product was cloned into the EcoRI and SmaI sites of pCC1, thus generating pCCΔ*ifmA*. The *swrAA* deletion mutant (PB5334 in Table 1) was

TABLE 2. Primers used in this study

Primer	Sequence[a]	Restriction enzyme
vjB-F	ggGGTACCAATGGGGGACGGCAGCAAC	*Kpn*I
vjB-R	ggcCTCGAGTCTCTCTCATATATTGATATC	*Xho*I
vzD-F	cgGAATTCTAACGAGTTAATATGCA	*Eco*RI
vzD-R	gcGGATCCGCGCTTTTTAACAGTTCAATTCC	*Bam*HI
A-rev	accgCTCGAGTTGTGAACCCCCATTTTCTTTATACAGATAAGCAC	*Xho*I
D-for	cgGGATCCGAAATTATCGGTTTGAATGGAG	*Bam*HI
E-rev	ataagaatGCGGCCGCTTCATGTCAGCCAGCAAC	*Not*I
Up-PromA	ccGAATTCTTTGTGCTTAAAGAGATTATGGATC	*Eco*RI
yvjD2	acgGAATTCATTATGATCCCGAAGCGAC	*Eco*RI
yvzDF1	TTGTGCGTGAAAAAAAAATAT	
yvjDR2	GTCTTCACGGTCATCCTTTAC	
yvjBF1	CCAATGGGGGACGGCAGCAAC	
yvzDR1	TCTCTCTTGCGTCATCCACAG	
yvzD1	ctcGGATCCTTGAAGAGGGCAAGTATTGTG	*Bam*HI
yvzD2	acgGAATTCTTATTATCTCTCTTGCGTCATCC	*Eco*RI
yvjD3450	acgGAATTCTTATTAGTTATGCTGAAGCGCTTCATAG	*Eco*RI
PGEXinter	acgGAATTCACTATCTCTCACCGCCTCAAGC	*Eco*RI
pS92	acgGAATTCTGCGCATGTGAGAACATCAC	*Eco*RI
PGEXPepSig	acgGAATTCAGTCCCTTTGTATATGTAAATTTCAG	*Eco*RI
pDH3	cccAAGCTTGTGTCTGTTCAATGGGGAATTG	*Hind*III
pDH4	acatGCATGCGCAAGTAAACCGAGAGGAATC	*Sph*I
pS287	acgGAATTCCATAAAGCAAAGAGTGAATG	*Eco*RI

[a] Lowercase letters indicate a specific tail; underlining indicates the restriction sequence.

produced by transformation of PB5249 with the linearized form of pCCΔ*ifmA* and by selection for kanamycin resistance.

The plasmid pCCΔ*ifmA*Δ*ifmB* was constructed for producing a PB5249 derivative strain carrying a deletion of both *swrAA* and *swrAB*. PCR amplifications were carried out with PB5249 chromosomal DNA by using primers D-for (BamHI site) and E-rev (NotI site) (Table 2) to amplify a region containing the last 130 bp of *swrAB* and the following 470 bp and with pCCΔ*ifmA* by using primers vzD-F and vzD-R. After BamHI digestion, the two fragments were ligated and inserted into the EcoRI/NotI sites of pCC1, generating plasmid pCCΔ*ifmA*Δ*ifmB*. After linearization, pCCΔ*ifmA*Δ*ifmB* was used to transform PB5249. Selection of transformants for kanamycin resistance led to the isolation of strain PB5336 (Table 1).

For *amyE* complementation experiments, *swrAA* and part of its upstream region were amplified by using primers Up-PromA and yvzD2 (each with an EcoRI site) (Table 2). The amplification was carried out with chromosomal DNA of PB5249, and the amplified fragment was digested with EcoRI and inserted into the EcoRI restriction site of pJM116 (31), which carries a chloramphenicol resistance marker and polylinker between the arms of the *amyE* gene, thus generating pCC16-8A. The plasmid was linearized by PstI digestion and used to transform PB5336. Transformants were selected for chloramphenicol resistance, and disruption of *amyE* was verified (strain PB5349 in Table 1). The same procedure was followed to construct plasmid pCC16-8AB that carries the region containing *swrAA* and *swrAB*. Amplification of the region was carried out with chromosomal DNA of PB5249 by using primers Up-PromA and yvjD2 (each with an EcoRI site) (Table 2). PB5336 was transformed with the linearized form of pCC16-8AB, and strain PB5369 (Table 1) was selected for chloramphenicol resistance and *amyE* disruption.

To place the *swrAB* coding sequence under control of the IPTG-inducible P*spac* promoter, chromosomal DNA of PB5249 was amplified with primers pDH3 (HindIII site) and pDH4 (SphI site) (Table 2). After HindIII and SphI digestion, the fragment was ligated to HindIII- and SphI-restricted pDH87 (31), thus producing pDH-ifmB. The plasmid was used to transform PB5249, and transformants were selected for chloramphenicol resistance.

Motility assays. Swimming motility was evaluated by seeding stationary-phase cells (5 μl; 2.0×10^8 cells ml^{-1}) onto the centers of 5-cm-diameter motility plates (swim plates; TrB was added with 0.2% [wt/vol] agar). Plates were incubated at 37°C, and the diameters of halos due to bacterial migration were measured 6 h postinoculation. Swimming in liquid media was also evaluated under a phase-contrast microscope (BH-2; Olympus) by observing the smooth swimming or tumbling phenotype exhibited by bacteria suspended in a drop of LB broth.

Phenotypic assays for swarming were initiated by spotting 2 μl of an overnight culture at the centers of tryptone-NaCl plates containing 1.0% agar (TrA). Plates were incubated for up to 24 to 48 h at 37°C, and swarm cell differentiation was analyzed as previously described (36, 37). For microscopic examination of the cells, samples were obtained by slide overlaying on isolated colonies. Flagellar filaments were stained with tannic acid and silver nitrate, followed by fucsin staining (36). Flagella appeared very fragile and could be better seen in microscopic fields containing isolated cells. To measure the cell lengths, the same transfer procedure was used but bacteria were Gram stained. For each strain, at least 100 microscopic fields were observed. The extent of cell flagellation after growth on TrA for 6 h was measured by harvesting cells from plates with cold water. Cell suspensions were normalized at an optical density at 600 nm (OD_{600}), vortexed, and centrifuged at $5,000 \times g$ for 15 min at 4°C. Flagellar filaments were collected from supernatants by high-speed centrifugation at $100,000 \times g$ for 1 h and subjected to protein gel electrophoresis. Motility assays were performed at least three times on separate days.

RNA isolation and RT-PCR. Total RNA was purified from *B. subtilis* cultures grown in TrB or TrA for 6 h as previously described (37). An RNA aliquot was examined on agarose gel to ensure its integrity. Reverse transcription-PCRs (RT-PCRs) were performed in one-step reactions. Up to 1 μg of RNA was mixed with 0.8 μM (each) primers in avian myeloblastosis virus (AMV)/Tfl buffer (50 mM Tris-HCl [pH 8.3], 50 mM KCl, 10 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM spermidine) containing 1.0 mM $MgSO_4$, 0.1 mM deoxynucleoside triphosphate, 25 U Tfl polymerase (Promega), and 3.75 U AMV reverse transcriptase (Promega) at a final volume of 25 μl. The following primers were used: yvzDF1, yvjDR2, yvjBF1, and yvzDR1 (Table 2). Contamination by DNA was excluded by carrying out reactions without the addition of the AMV reverse transcriptase. Positive controls were obtained using genomic DNA as a template.

Preparation of recombinant SwrAA and SwrAA-specific antiserum. Recombinant SwrAA fused with glutathione *S*-transferase (GST) was produced in *E. coli* BL21. Primers yvzD1 (BamHI site) and yvzD2 (EcoRI site) (Table 2) were used to amplify *swrAA* from PB5249. The amplification product was inserted into the BamHI/EcoRI sites of pGEX-6P-1 (Amersham Biosciences). Recombinant *E. coli* clones were grown in LB broth containing ampicillin to an OD_{600} of 0.9; IPTG was added, and cultures were grown for a further 3 h. Cells were collected by centrifugation, suspended in phosphate-buffered saline, and sonicated. Triton X-100 was added to a final concentration of 0.1%, and cell lysates were collected after centrifugation at $21,000 \times g$ for 15 min at 4°C and incubated with glutathione Sepharose 4B beads (Amersham Biosciences). After extensive washing, beads were treated with PreScission protease (Amersham Biosciences) and SwrAA was recovered. Protein samples were taken during the procedure and inspected for yield and purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). BALB/c mice were immunized by intraperitoneal injection with recombinant SwrAA (50 μg in Tris-HCl buffer) emulsified in Freund's incomplete adjuvant (1:1). At day 15, the mice received a booster injection (50 μg of the protein in adjuvant), and serum samples were obtained after 72 h.

Expression of *B. subtilis swrA* in *E. coli*. *E. coli* BL21 was used as the recipient for all protein expression experiments, as described above. A DNA fragment comprising *swrAB* was produced by amplification of chromosomal DNA extracted from PB5249 by using primers yvzD1 (BamHI site) and yvjD2 (EcoRI site) (Table 2). After EcoRI-BamHI digestion, the fragment was inserted into pGEX-6P-1, in frame to the GST gene, thus generating plasmid pX-AB. This plasmid was used to produce both the fusion protein GST-SwrAA and SwrAB. To obtain coexpression of GST-SwrAA and SwrAB carrying various kinds of mutations, the plasmids pX-ABnter, pX-ABcter, pX-PDZ, and pX-ABΔTD1 were produced. pX-ABnter is derived from pGEX-6P-1 after insertion into the BamHI/EcoRI sites of a DNA fragment produced by amplification of PB5249 chromosomal DNA with yvzD1 and yvjD3450 (EcoRI site) (Table 2) containing *swrAB* lacking the last 144 bp. pX-ABcter was used to express the fusion protein GST-SwrAA and SwrAB from which the region between the 2nd and the 78th amino acids had been deleted. A DNA segment corresponding to *swrAA* and the intergenic region between *swrAA* and *swrAB* was amplified by using primers yvzD1 and PGEXinter (EcoRI site) (Table 2). This fragment was cloned into the BamHI/EcoRI sites of pGEX-6P-1, producing pGEXinter. Part of *swrAB* was amplified by using primers pS92 (EcoRI site) and yvjD2 (Table 2) and inserted into the EcoRI site of pGEXinter, thus producing pX-ABcter. To obtain pX-PDZ for the coexpression of the fusion GST-SwrAA and the PDZ domain of SwrAB, a DNA fragment corresponding to the PDZ domain was amplified by using primers pS287 (EcoRI site) (Table 2) and yvjD2 and inserted into the EcoRI site of pGEXinter. pX-ABΔTD1 was used to coexpress the fusion protein GST-SwrAA and SwrAB from which the region between residues 64 and 78 had been deleted. A DNA segment corresponding to *swrAA*, the intergenic region between *swrAA* and *swrAB*, and part of *swrAB* was amplified by using primers yvzD1 and PGEXPepSig (EcoRI site) (Table 2). After EcoRI-BamHI digestion, the fragment was inserted into the BamHI/EcoRI sites of pGEX-6P-1, yielding plasmid pGEXPepSig. Part of *swrAB* was amplified by using primers pS92 and yvjD2 (Table 2) and inserted into the EcoRI site of pGEXPepSig, generating pX-ABΔTD1. During the cloning procedure, the 64th residue (Trp) of SwrAB was changed into Arg, thus producing plasmid pARN64.

Preparation of *B. subtilis* cell lysates. *B. subtilis* strains were grown in TrB or TrA for 6 h at 37°C. Cells were harvested by washing the surfaces of agar plates with cold water and normalized with respect to the OD_{600}s of liquid cultures. Cell suspensions were centrifuged at $5,000 \times g$ for 15 min at 4°C, and the bacterial pellets were washed with cold Tris-buffered saline (pH 7.4) and lysed in 1 ml Tris-buffered saline with zirconium beads (diameter, 0.1 mm) by shaking at 4°C for 4 min with a mini-bead beater (BioSpec Products, Bartlesville, OK). Residual cells and debris were removed from the lysate by centrifugation at $12,000 \times g$. Cell lysates were concentrated with Microcon YM-3 filters (Millipore, Bedford, MA), stored at −20°C, and used within 1 week.

Effect of *B. subtilis* plasma membranes on swrAA. To obtain *B. subtilis* plasma membranes, cells were grown in 50 ml of LB broth, harvested at an OD_{600} of 0.5, washed in 10 mM potassium-phosphate buffer, pH 6.4, and suspended into 10 ml of 10 mM potassium-phosphate buffer containing 25% sucrose and 2 mg ml^{-1} lysozyme. Suspensions were incubated at 37°C with gentle shaking for 1.5 h. Protoplast formation was assessed by microscopic inspection of samples taken at several time points during incubation. Samples were centrifuged at $4,500 \times g$ for 15 min at 4°C, and protoplasts were lysed by suspending the sediment in 1 ml of ultrapure water and then centrifuged at $20,000 \times g$. The pellet was suspended in 20 μl of Tris-HCl (10 mM; pH 7.2) and added together with recombinant SwrAA (20 μg). After incubation for 1 or 2 h at 37°C, the samples were electrophoresed. A negative control was constituted by incubating SwrAA at 37°C in Tris-HCl (10 mM; pH 7.2) for the same time periods. Experiments were performed three times on separate days.

Protein gel, immunoblot, and MALDI-TOF analyses. Protein samples were suspended in sample buffer containing β-mercaptoethanol, heated at 95°C for 10

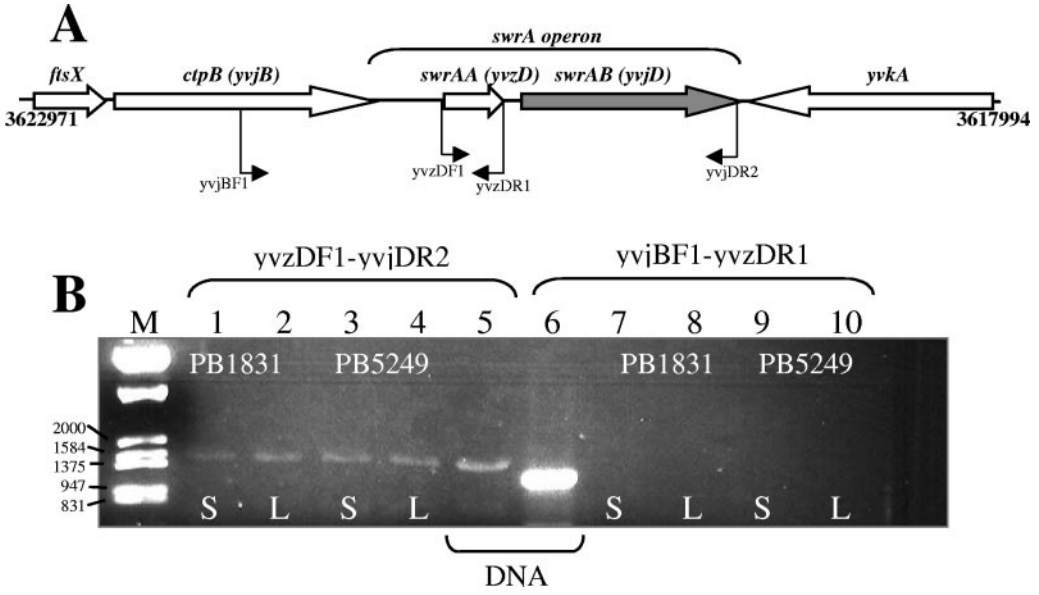

FIG. 1. *B. subtilis swrA* operon. (A) Genetic organization of the *swrA* operon. (B) Amplification products obtained by RT-PCR on total RNA from strains PB1831 and PB5249, grown in solid (S) and liquid (L) media, by using primers yvzDF1-yvjDR2, designed to amplify an *swrAA-swrAB* overlapping region (lanes 1 to 4), and yvjBF1-yvzDR1, designed to amplify a *yvjB-swrAA* overlapping region (lanes 7 to 10). PCR amplifications of chromosomal DNA with primers yvzDF1-yvjDR2 (lane 5) and yvjBF1-yvzDR1 (lane 6). M, molecular size standard.

min, and separated by SDS-PAGE; gels were either silver stained or used for protein blotting. Immunolabeling on Western blots was carried out by using antibodies raised against *B. subtilis* flagellin or against recombinant SwrAA. Matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) analysis was carried out as previously described (10).

RESULTS

Characterization of the *ifmP* mutant of *B. subtilis*. A recently described hypermotile mutant, isolated from the laboratory strain PB1831, was characterized by an increased number of flagella when grown in liquid culture and by the ability to switch from swimming to swarming when being transferred from liquid onto solid media (37). The mutant strain, PB5249, was found to carry a mutation, named *ifmP*, at approximately 310° on the *B. subtilis* chromosome. In order to identify the *ifmP* mutation, a wide chromosomal DNA region, between the genetic markers *hag* and *uvrA* and comprising the *ifm* locus, was sequenced. Sequences revealed a single difference in the *yvzD* gene: PB1831 was characterized by the insertion of one A · T residue in a short homopolymeric A · T tract, which has eight A · T base pairs (from nucleotide 3,620,660 to nucleotide 3,620,668) in PB5249. Thus, the mutation we detected in the *ifmP* mutant of *B. subtilis* corresponds to that recently described in *yvzD* as being responsible for a phase variation mechanism involved in *B. subtilis* swarming motility (21). The *yvzD* gene, which was referred to as *swrA* by Kearns and colleagues (21), has been here renamed *swrAA* (see below). Consequently, as suggested for many *B. subtilis* laboratory strains, PB1831 was regarded as the mutant, since it harbors a 9-bp A · T sequence and exhibits impairment in swarming motility, while PB5249, described as a *B. subtilis ifmP* mutant (37), was regarded as the wild-type strain, since it regained the ability to swarm.

Analysis of the 117-residue polypeptide encoded by *swrAA* (Swiss-Prot accession no. O32266) did not reveal any significant similarity with sequences present in data banks, except with an almost identical sequence (85.5% identity and 93.2% similarity) found in the recently sequenced genome of *Bacillus licheniformis* (Swiss-Prot accession no. Q62PV3). In addition, while an average of 56.5% A+T content has been estimated for *B. subtilis* (23), an unusually high A · T-rich island was evident over the length of the *swrAA* coding sequence and that of the upstream intercistronic region, with the A+T content being 66.6%.

The *swrAA* sequence with eight A · T base pairs was cloned into the pJM114 integrative vector (31) and introduced into the chromosome of strain PB1831, thus generating PB5340 (Table 1). Analysis of phenotypic traits exhibited by the transformants showed that in the presence of a functional copy of *swrAA* the ability to swarm was regained (data not shown).

Identification of a dicistronic operon, *swrA*, required for the extent of cell flagellation and for swarming differentiation. In the published sequence of the *B. subtilis* chromosome (23), the coding sequence of *swrAA* is separated from the upstream gene *ctpB* (*yvjB*) by a 385-bp intercistronic region and from the downstream gene *yvjD* by an 81-bp region; *yvjD* is followed by *yvkA*, which is transcribed in the opposite direction (Fig. 1A). To evaluate whether *swrAA* was cotranscribed with *yvjD* and *ctpB*, RT-PCR analysis was carried out by using RNA purified from PB5249 and PB1831. Amplification was obtained when primers yvzDF1 and yvjDR2, designed on the sequences of *swrAA* and *yvjD*, respectively, were used, thus giving evidence of unique transcripts for these genes in both strains that were independent of the growth conditions adopted (Fig. 1B, lanes 1 to 4). Since no amplification product was obtained with primers yvjBF1 and yvzDR1, designed on the sequences of *ctpB* and *yvzD*, respectively (Fig. 1B, lanes 7 to 10), we concluded that *yvzD* and *yvjD*, but not *ctpB*, constitute a dicistronic

operon, which we named *swrA*, with *yvzD* and *yvjD* referred to as *swrAA* and *swrAB*, respectively. The entire operon is transcribed in cells grown in either liquid or solid media.

The *swrAB* cistron. The nucleotide sequence of *swrAB* (Fig. 2A) was found to share significant similarity with sequences coding for putative transmembrane proteins of *Bacillus cereus* (36% identity; 59% similarity) and *Bacillus anthracis* (35% identity; 59% similarity) and lower similarity with other putative proteins from a number of bacilli (Fig. 2B). Since none of these proteins has been characterized, inferences regarding a possible role for *swrAB* were not possible. The deduced amino acid sequence of SwrAB shows the following main features: (i) a putative N-terminal signal peptide; (ii) multiple transmembrane domains; and (iii) a conserved protein-protein interaction module PDZ (32) at the C terminus of the protein, extending from residue 295 to residue 361 (Fig. 2A). The PDZ domain is of approximately 90 amino acid residues, has a compact globular structure, and is thought to function in organizing signal transduction complexes and in clustering membrane receptors (29, 33, 35). The presence of a PDZ module and multiple transmembrane domains suggests that SwrAB may be involved in protein-protein interaction (29) at the interface of the plasma membrane (33), potentially enabling the recruitment and organization of protein complexes involved in signal transduction and/or in sensing of cytoplasmic proteins (35).

The demonstration that *swrAA* is part of a dicistronic operon prompted us to explore the role played by *swrAA* and *swrAB* in the control of *B. subtilis* flagellum-dependent motility as well as in the initiation of swarm cell differentiation.

Functional analysis of the *B. subtilis swrA* operon. The functional contribution of the two cistrons, *swrAA* and *swrAB*, in the control of cell flagellation and swarming differentiation was assessed by examining the phenotype exhibited by PB5249 derivatives generated by deletion of *swrAA* (PB5334) or of the entire *swrA* operon (PB5336), followed by complementation in the *amyE* locus. The different strains were characterized on the basis of colony morphology, level of cell flagellation upon growth on 1% agar plates, and amount of extracellular flagellin, as assessed by Western blotting of protein from liquid- and surface-grown cultures (Fig. 3). In addition, the presence of elongated swarm cells was evaluated by microscopy with Gram-stained cells collected from the growing colonies (Table 3).

The phenotypes of the *swrAA* (PB5334) and *swrA* (PB5336) null mutants were similar to that described for PB1831 (37). Colonies were rough and curled (Fig. 3), formed by chains of short cells (Table 3) that did not synthesize flagella (Fig. 3). Thus, PB5334 and PB5336 did not mount a swarming response when grown on swarm plates (TrA). Both strains were significantly less motile than the wild-type strain PB5249 when propagated on swim plates (Fig. 4). These observations indicate that strains either harboring a 9-bp A · T *swrAA* allele (PB1831) or completely lacking *swrAA* behave similarly. Furthermore, the observation that deletion of the entire *swrA* operon, in strain PB5336, does not further affect the phenotype of strains lacking *swrAA* (PB5334) on either swarm (Fig. 3) or swim (Fig. 4) plates did not address the role played by *swrAB* either in flagella assembly or in swarming differentiation. The phenotype exhibited by a mutant defective in the *swrAB* open reading frame could not be analyzed, since deletion of the sole *swrAB* open reading frame was lethal. Interestingly, a similar lethal effect was observed upon introduction of a point mutation in *swrAB* (see below and legend to Fig. 6A). Additional circumstantial evidence for the essentiality of *swrAB* was obtained by placing this gene under the control of the IPTG-inducible P*spac* promoter. To this end, a fragment of the *swrAB* coding sequence was cloned downstream of P*spac* in the integrative plasmid vector pDH87 (31). The sequence included the GTG start codon and extended through approximately one-fifth of the gene. Transformants were obtained but could be maintained only in the presence of IPTG; in the absence of an inducer, the cells lysed. In contrast, both deletion of the entire *swrA* operon and ectopic reintroduction of *swrAA* into the *amyE* locus in PB5336 were tolerated. An explanation for this phenomenon was not experimentally obtained. However, we supposed that a perfectly tuned level of expression of both gene products could play a crucial role in preventing the lethal effect that *swrAA* apparently produces when expressed alone.

A hint about the possible role of *swrAB* stemmed from the observation that the defective phenotype of the double-deletion strain PB5336 was only partially rescued by complementation with *swrAA* in the *amyE* locus (PB5349). Cells showed an increase in the degree of cell flagellation in liquid media, regained the ability to synthesize flagellar filaments when grown on solid surfaces (Fig. 3), and were found to swim more rapidly than PB5336 cells (Fig. 4). Colonies were similar to those of the wild-type PB5249, i.e., they had a glossy appearance, with compact groups of cells protruding from the main body of the colony (Fig. 3). Interestingly, PB5349, although flagellated, did not undergo swarm cell differentiation, as shown by the lack of hyperflagellation (Fig. 3) and elongation (Table 3) of cells growing on swarm plates. Conversely, *amyE* complementation with the entire *swrA* operon (PB5369) restored both flagellation and swarming in a PB5336 double mutant (Fig. 3 and Table 3). From these results, we conclude that *swrAA* plays a crucial role in regulating the degree of cell flagellation, in both liquid and solid media, but it is not sufficient to enable surface-adhering cells to differentiate into hyperflagellated and elongated swarm cells. The differentiation process requires the activity of *swrAB*.

Interaction between SwrAA and SwrAB. As shown in Fig. 5, SwrAA was detectable in cytosolic cell fractions from PB5249, which harbors the wild-type *swrA* operon. The protein was present in cells from broth culture and agar plates.

SwrAB is a putative membrane-bound protein that contains a PDZ domain (see the legend to Fig. 2A for details), which is thought to confer protein binding capability. In *B. subtilis*, the PDZ domain identified in SpoIVB is the most extensively studied and proven to confer multiple regulatory functions on SpoIVB (5, 17, 18, 30). To evaluate whether an interaction between SwrAA and SwrAB occurs, *swrAA* alone and the entire *swrA* operon, with *swrAA* fused to the GST coding sequence, were cloned and expressed in *E. coli* BL21 (Fig. 6A). Following induction with IPTG, on gels with whole-cell homogenates from *E. coli* BL21 transformed with *swrAA* alone, one protein band was clearly prominent at a molecular mass of approximately 40 kDa, as expected for full-length SwrAA fused to GST (Fig. 6B, lane 1). In sharp contrast, in whole-cell homogenates from *E. coli* transformed with the entire *swrA*

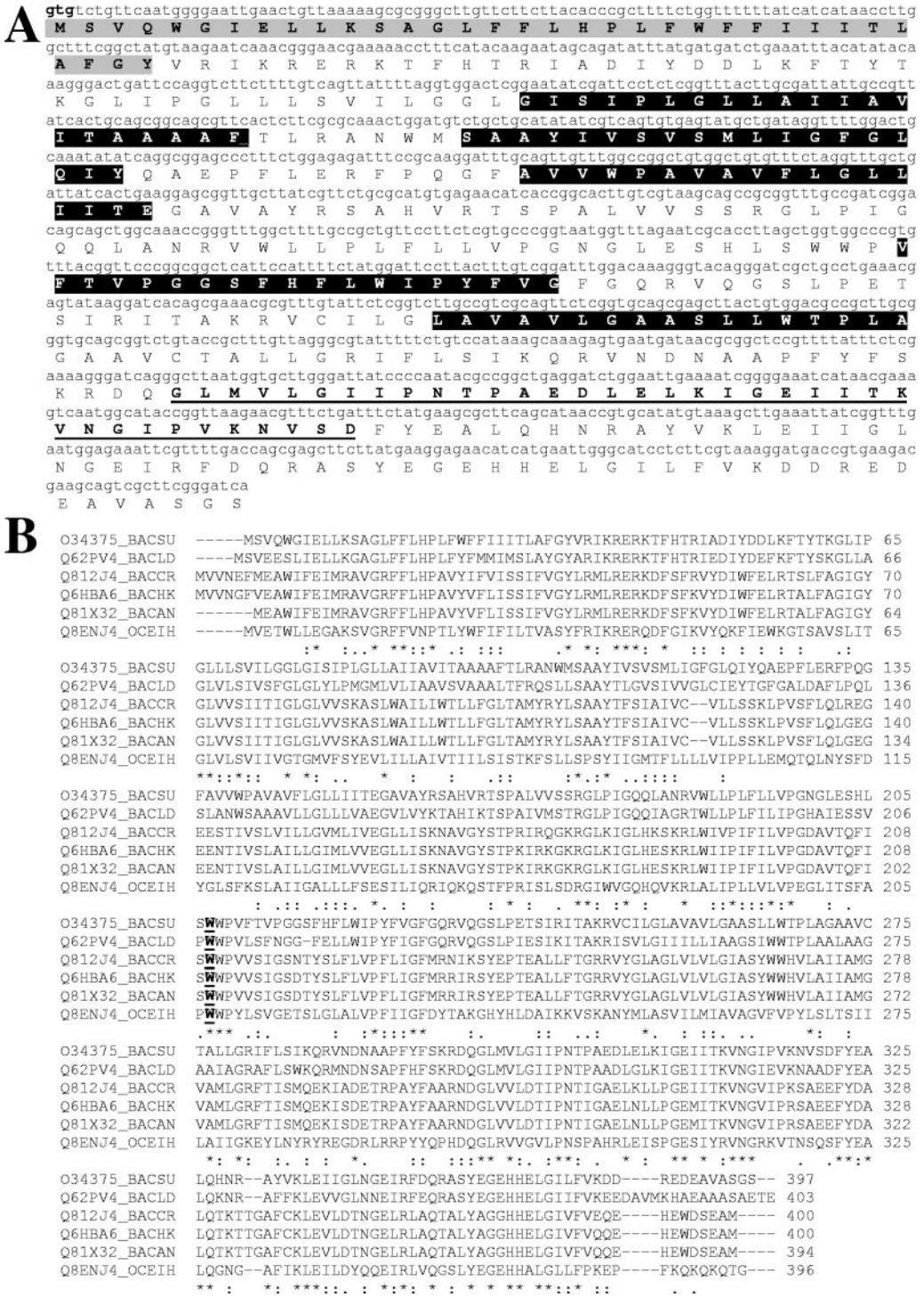

FIG. 2. *B. subtilis swrAB*. (A) Nucleotide (lowercase letters) and deduced amino acid (uppercase letters) sequences of *swrAB*. The *swrAB* putative signal peptide sequence is in gray boxes, the putative transmembrane domains are in black boxes, and the PDZ domain is boldfaced and underlined. (B) ClustalW alignment of proteins similar to SwrAB from different bacilli. Asterisks indicate identical residues, colons indicate conserved substitutions, and dots indicate semiconserved substitutions. The conserved W necessary for SwrAB function is boldfaced and underlined. Swiss-Prot accession numbers, given at the left, refer to the following species: O34375_BACSU, *B. subtilis*; Q62PV4_BACLD, *B. licheniformis* ATCC 14580; Q812J4_BACCR, *Bacillus cereus* ATCC 14579; Q6HBA6_BACHK, *Bacillus thuringiensis* serovar Konkukian; Q81X32_BACAN, *B. anthracis* strain Ames Ancestor; and Q8ENJ4_OCEIH, *Oceanobacillus iheyensis* HTE831.

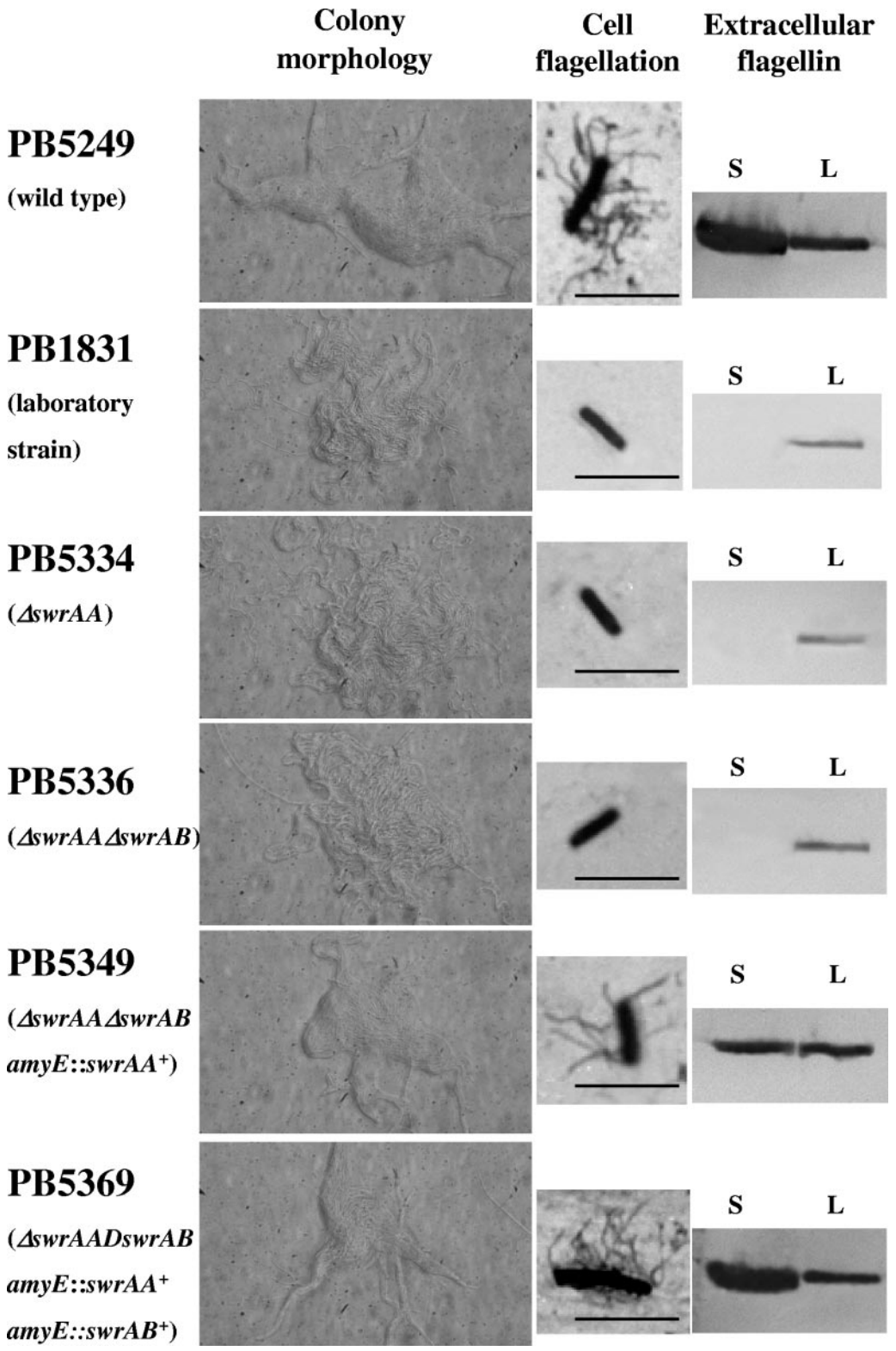

FIG. 3. Morphological traits of the wild-type *B. subtilis* PB5249, the laboratory strain PB1831, and PB5249 derivatives carrying a deletion of either *swrAA* (PB5334) or *swrA* (PB5336). Stains PB5349 and PB5369 derive from PB5336 upon complementation (in the *amyE locus*) with a functional copy of *swrAA* (containing an 8-bp A-T stretch) and a functional copy of *swrA* (containing an 8-bp A-T *swrAA* sequence), respectively. Panels show colony morphology (left) and cell flagellation (center) after 6 h of growth on swarm plates; extracellular flagellin (right panel) from cells grown in solid (S) and liquid (L) media was detected by Western blotting. *DswrAB*, Δ*swrAB*. Bars, 5 μm.

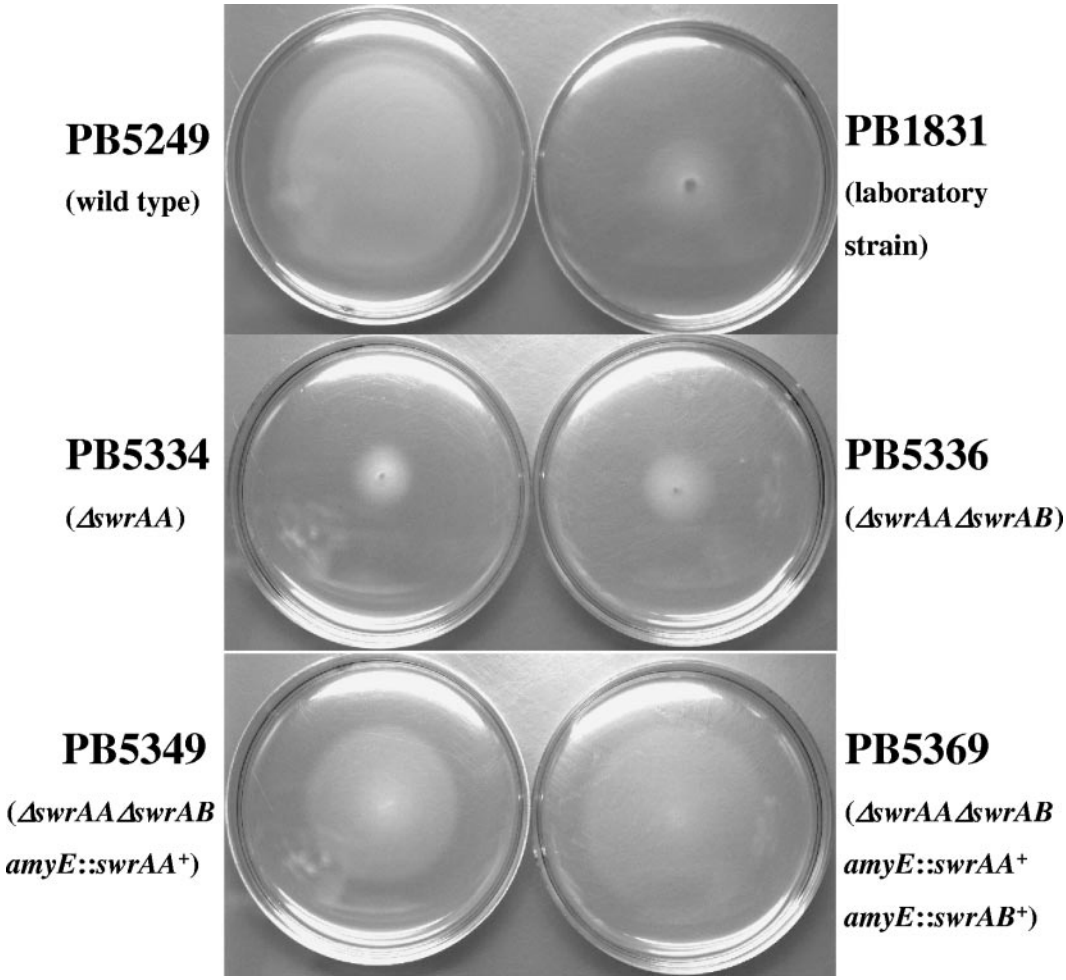

FIG. 4. Swimming motilities of different *B. subtilis* strains.

operon, no band with the expected mobility of GST-SwrAA or SwrAB (44 kDa) was detected, while one prominent band appeared with a mobility similar, but not identical, to that of the GST tag (Fig. 6B, lane 2). This protein could be purified on glutathione Sepharose beads, proving to be indeed a GST derivative (Fig. 6C, lane 2). MALDI-TOF mass spectroscopy analysis of this protein gave a mass of 28.829 kDa, 746 Da higher than that expected for the sole entire GST but compatible with that of GST fused to the first 18 amino acid residues of SwrAA. These data suggest that SwrAB leads to hydrolysis of SwrAA, as SwrAA is not degraded in the absence of SwrAB. However, in order to exclude artifacts, the coexpression experiment was performed using a *swrAB* spontaneous mutant carrying an amino acid substitution, arginine instead of tryptophan, at position 208. The mutation was accidentally obtained during PCR amplification of the *swrAB* coding sequence. Coexpression of SwrAA and SwrAB carrying a point mutation did not lead to degradation but allowed detection of full-length GST-SwrAA (Fig. 6B, lane 7). These results strongly suggest that the observed cleavage of SwrAA occurs only in the presence of wild-type SwrAB (Fig. 6B, lane 2); moreover, since arginine-tryptophan substitution occurred in a highly conserved residue (Fig. 2B), we speculate that the residue might

TABLE 3. Lengths of swarm and nonswarm cells taken from colonies growing on swarm plates

Cell type	Length[a] (μm) of cells of *B. subtilis* strain					
	PB5249	PB1831	PB5334	PB5336	PB5349	PB5369
Nonswarmer	2–4	2–4	3–4	3–4	3–6	3–4
Swarmer	10–16	ND	ND	ND	ND	9–13

[a] ND, not detectable, i.e., cells longer than the nonswarmers were not observed in examinations of more than 100 microscopic fields (magnification, ×1,000).

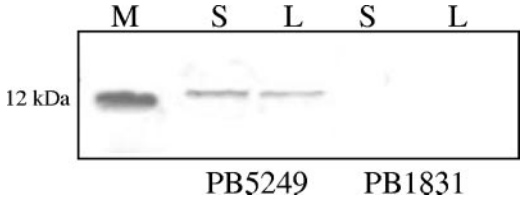

FIG. 5. Results of immunoblotting of cell lysates from strains PB5249 and PB1831, grown on solid (S) and in liquid (L) media, with an anti-SwrAA antiserum. M, molecular size standard.

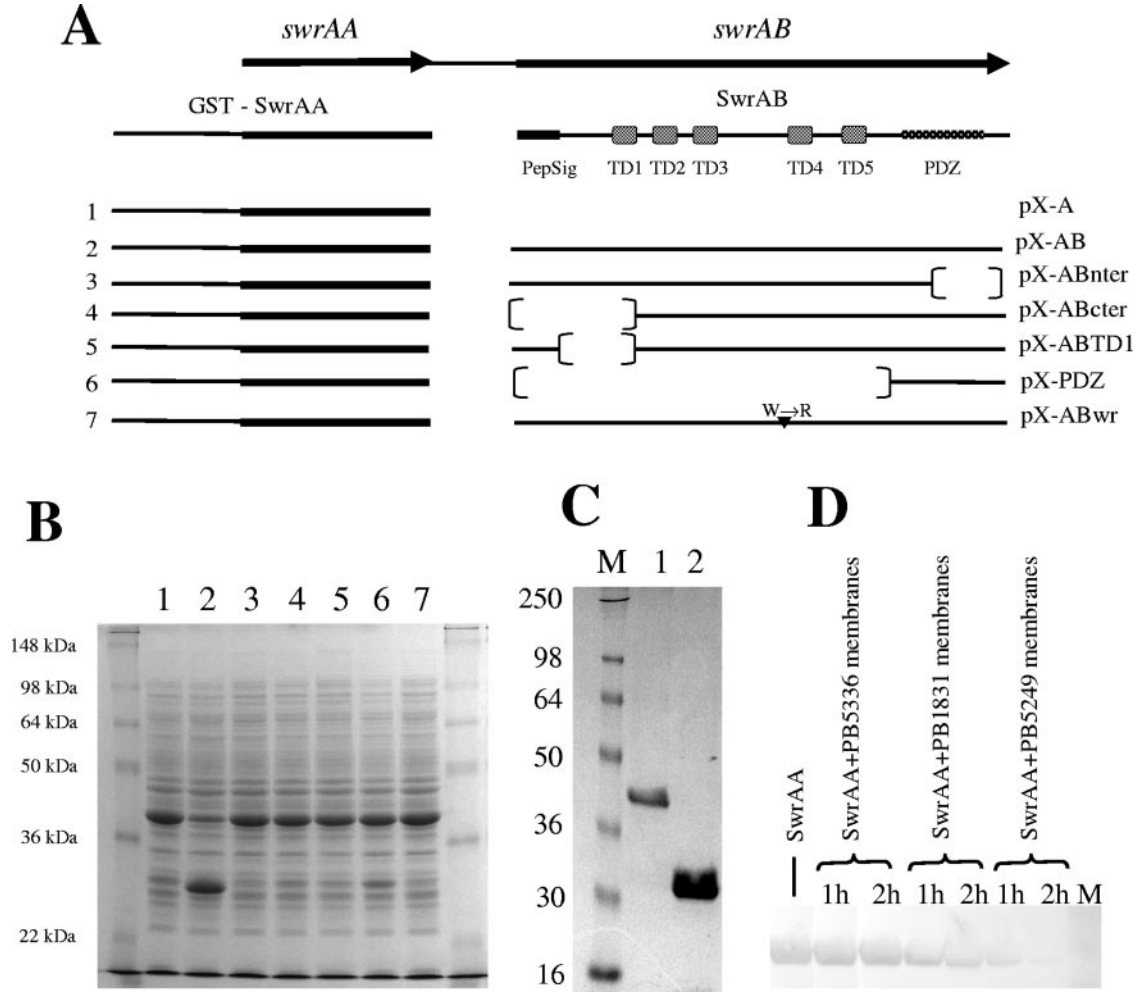

FIG. 6. Expression of GST gene-*swrA* and GST gene-*swrAA* fusions in *E. coli*. (A) Diagram showing the *swrA* operon and the gene fusions. Constructs were as follows: 1, GST gene-*swrAA* fusion; 2 through 7, GST gene-*swrA* fusions with either native *swrAB* (2) or mutated *swrAB* carrying a deletion corresponding to a portion of the PDZ domain (3), the first transmembrane domain (TD1) plus the signal peptide (PepSig) (4), the first transmembrane domain only (5), or transmembrane domains 1 through 5 and the signal peptide (6) or harboring a point mutation producing a change from tryptophan (W) to arginine (R) (7). (B) SDS-PAGE of whole-cell homogenates from *E. coli* clones transformed with constructs 1 through 7. (C) SDS-PAGE of purified GST-SwrAA fusion proteins obtained with constructs 1 and 2. (D) Western blot of the same amounts of purified SwrAA incubated at 37°C for 1 or 2 h without and with plasma membranes isolated from strains PB5336 (Δ*swrAA* Δ*swrAB*), PB1831 (laboratory strain carrying a 9-bp A · T stretch of *swrAA*), and PB5249 (wild-type strain carrying an 8-bp A · T stretch in *swrAB*). M, molecular size standard.

be indispensable for SwrAB activity. Overproduction of SwrAB alone was attempted, but probably due to the multiple transmembrane regions present, this protein cannot be produced in *E. coli*.

Attempts to identify the region of SwrAB involved in SwrAA degradation were performed by expressing GST-SwrAA together with a different portion of SwrAB (Fig. 6A and B). In each case, full-length GST-SwrAA was observed (Fig. 6B, lanes 3 to 5) as occurred with SwrAB carrying a point mutation (lane 7). These observations, although carried out in an artificial environment, support the view that (i) full-length SwrAA is no longer detectable in the presence of wild-type SwrAB, (ii) SwrAA is not cleaved in the absence of functional SwrAB, and (iii) the catalytic activity of SwrAB requires the presence of the entire protein. The last point is also supported by preliminary results. A C-terminal part of SwrAB, corresponding to the complete PDZ domain, was efficiently expressed in *E. coli* as a fusion with GST; the insoluble fusion product was solubilized with 8 M urea. We did not observe any modification of SwrAA upon incubation with the renatured GST-fused PDZ region (data not shown).

As the expression and purification of SwrAB alone was not possible in *E. coli*, we used *B. subtilis* plasma membranes, as a source of SwrAB, and purified recombinant full-length SwrAA to evaluate whether degradation of SwrAA could be detected in an in vitro assay. A fixed amount (15 μg) of purified recombinant full-length SwrAA was incubated with *B. subtilis* plasma membranes isolated from the wild-type PB5249 and from PB1831. Membranes from PB5336, which carries a deletion of the entire *swrAB* operon, were used as a negative control. A marked reduction of the SwrAA band, as detected by Western blotting, occurred only in the presence of plasma membranes

derived from PB5249 (Fig. 6D, lanes 6 and 7) and, to a lesser extent, from PB1831 (Fig. 6D, lanes 4 and 5). These preliminary data are in line with a potential interaction between SwrAA and the membrane-bound SwrAB leading to SwrAA processing. However, as SwrAA is detectable in the soluble cell fraction of PB5249 (Fig. 2), which harbors the entire operon, it may be hypothesized that SwrAA degradation is under the control of not-yet-identified cellular factors. Further experiments will be necessary to establish whether a full swarming phenotype requires SwrAB, a processed form of SwrAA, or both.

DISCUSSION

The degree of cell flagellation in eubacteria is a characteristic phenotypic trait of each bacterial species. The number and distribution of flagellar filaments exhibited at the cell surface are successfully maintained over generations and are frequently used as an additional taxonomic key to distinguish bacterial species that share common physiological features. Nothing is known about genes and/or molecular mechanisms regulating the amount of cell flagellation in *B. subtilis*, although it has long been understood that the *ifm* locus of *B. subtilis* influences the number of flagella in liquid media and the motility phenotype of this peritrichous microorganism (11).

Our major finding was the discovery of a *B. subtilis* operon, *swrA*, which controls the assembly of flagella and regulates swarming differentiation in response to bacterial contact with solid surfaces. The *swrA* operon is dicistronic, and *swrAA* and *swrAB* genes are expressed by cells growing both in liquid and in solid media. Functional analysis of the *swrA* operon, assessed by using deletion mutations followed by complementation in the *amyE* locus, allowed better definition of the roles played by *swrAA* and *swrAB*, respectively, in the control of *B. subtilis* flagellum-dependent motility and in the initiation of swarm cell differentiation. Complementing the *swrA* deletion strain with the functional *swrAA* cistron alone, we demonstrated that *swrAA* controls the degree of flagellation in liquid media and governs the assembly of flagellar filaments in response to solid surfaces but fails to initiate swarming differentiation of surface-adhering cells. Thus, we concluded that (i) *swrAA* is the first characterized *B. subtilis* gene that acts as a regulatory gene governing the number of functional flagellar filaments and is necessary for enabling surface-adhering cells to assemble flagella and that (ii) the novel discovered gene, *swrAB*, is needed by the surface-adhering cells to differentiate into swarmers. Direct evidence that *swrAB* plays such a central role in swarm cell differentiation could not be found in strains lacking a functional *swrAA* allele. In fact, *swrAA*-defective cells are completely lacking flagella when grown over solid surfaces and, consequently, do not exhibit swarming behavior.

The clustering of *swrAA* and *swrAB* in a dicistronic operon appears to have an adaptive value: *swrAA* controls the extent of flagellation and *swrAB* governs swarming, which requires large numbers of flagellar filaments. The finding that flagellar number is subjected to a phase variation mechanism due to insertion/deletion of an A · T base pair should be considered in relation to the high percentage of A+T in the *swrAA* coding sequence. A+T-rich islands are usually considered to originate from bacteriophages or other insertion elements (23, 27), and it may be speculated that the capture of *swrAA* was successful as a controlling element: the gene provides a handy molecular base for phase variation (21) and, thus, has been preserved in wild-type *B. subtilis* strains. The functionality of the *swrA* operon plays an essential role in correctly eliciting an adaptive swarming response; however, it appears to be dispensable for the survival of *B. subtilis*. Swarming, in contrast to other bacterial differentiation processes, is not induced by nutrient restraints (8) and does not represent an additional physiological advantage to overcome periods of nutrient limitation and/or to survive environmental stress; rather, it is a proficient adaptive strategy allowing the expansion of growing bacterial communities when they meet nutrient-rich environmental surfaces.

The ability to mount a swarming response implies the sensing of external surfaces, the transduction of such a signal across the cell membrane, and the activation of different regulatory pathways leading the vegetative cells to initiate swarm cell differentiation. We began to explore how the two cistrons of the *swrA* operon act in regulating the extent of cell flagellation and the initiation of swarming differentiation in *B. subtilis*. Evidence has been produced that SwrAB has an effect on SwrAA; such an effect was shown to occur in *E. coli* and to determine proteolysis of SwrAA and appears to occur also in *B. subtilis*, as judged by the decrease in the amount of purified SwrAA observed upon its incubation with plasma membranes purified only from strains carrying an *swrAB* gene, in the presence and in the absence of endogenous *swrAA*, and not upon incubation with plasma membranes from PB5336, which lacks the entire *swrA* operon. SwrAB is a membrane protein that may be involved in the sensing of signals derived from growth on solid surfaces, and through its PDZ domain, it may participate in processing of SwrAA. Protein processing by cleavage is a common theme in regulatory processes that contribute to the developmental changes of bacterial cells (3, 17, 34).

Further investigation is required to understand the mechanism whereby SwrAA acts in regulating the degree of cell flagellation and the way in which the interaction between the membrane-bound SwrAB and SwrAA is involved in the induction of the adaptive swarming behavioral response.

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